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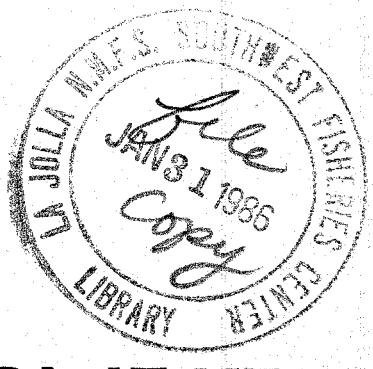

CIGUATERA AT MIDWAY: AN ASSESSMENT USING THE HOKAMA "STICK TEST" FOR CIGUATOXIN

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CIGUATERA AT MIDWAY: AN ASSESSMENT USING THE HOKAMA "STICK TEST" FOR CIGUATOXIN

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ABSTRACT

A simplified rapid immunoassay for detection of ciguatoxin in fish flesh recently has been developed by Yoshitsugi Hokama of the University of Hawaii. This "stick test" utilizes treated skewers which are inserted into the flesh of the fish. The sticks are then run through a series of solutions to produce a colorimetric indication of toxicity. The test could provide a means of preventing ciguatera seafood poisonings which are a problem throughout subtropical and tropical regions. A survey was undertaken to facilitate further development of this new test. The study was conducted at Midway in the Northwestern Hawaiian Islands (NWHI), an area which has experienced frequent outbreaks of fish poisonings.

Samples of 239 fish (34 species) were tested with the stick test. In addition, frozen flesh samples were returned to Honolulu for direct analysis of toxicity using the mouse bioassay. The results of the test can be summarized as follows:

1. Several modifications to the test were recommended. Many of the suggested changes have already been incorporated into the technique.

2. Results of the test were very encouraging despite the technical problems that might have reduced its accuracy. The stick test and the mouse bioassay gave negative results for species such as the mullet, Mugil cephalus, and the aholehole, Kuhlia sandvicensis, that are routinely eaten by the local population. In contrast, the parrotfish, Scarus perspicillatus, was shown extremely toxic by both the stick test and the mouse bioassay.

3. Inconsistent results also were noted. Discrepancies are believed to result from the presence of toxins and polyethers other than ciguatoxin in some of the fish. This problem is currently the focus of research in several laboratories.

INTRODUCTION

One of the responsibilities of the National Marine Fisheries Service (NMFS) is to ensure safe and wholesome seafood products. The consumer, the economy, and the fishing industry benefit from advances that enable us to better understand, detect, and prevent seafood poisonings. One of the most serious problems in subtropical and tropical insular regions has been that of ciguatera and related poisonings. Citizens of the States of Florida and Hawaii, the Territories of Puerto Rico, Guam, Virgin Islands, American Samoa, and the various developing Pacific island nations are threatened by this problem. The disease is characterized by gastrointestinal and neurological symptoms resulting from eating a variety of tropical marine fishes. Death can result in severe cases.

Recently, there has been a dramatic increase in the number of ciguatera poisonings in Hawaii. During the first 3 months of 1985, there were 44 poisonings (13 outbreaks) reported to the Hawaii State Department of Health, compared with 17 cases (3 outbreaks) for the same time period last year. The high rate of reported incidence is continuing in the second quarter. Department of Health records show many cases developing throughout the State at the present time and seafood restaurants as the source of some of the poisonings. Possible legal repercussions and economic impact as well as human health problems are apparent. This has been a troublesome and chronic problem. Solutions have been elusive even though considerable amounts of research have been expended at the international level.

Recently, several important research breakthroughs have been made. The source of the toxins is now known and the chemical structure of the toxin and mode of its action on the human body are being studied in more detail. New methods of detection are being developed. One of the most promising detection techniques is currently under development at the University of Hawaii by Yoshitsugi Hokama. The technique is an inexpensive, rapid, colorimetric immunoassay (Hokama in press). Obviously, development of an inexpensive, simple test for ciguatera could contribute substantially to the NMFS mission, so the Southwest Fisheries Center Honolulu Laboratory was very interested in applying this test in an actual field situation. Since this test is still in an early stage of development, trial use by the Honolulu Laboratory and others might identify problems and accelerate marketing of a successful test kit.

DESCRIPTION OF THE TEST

Briefly, this is a rapid, simplified enzyme immunoassay test for the detection of ciguatoxin and related polyethers from fish tissues. This test has been described in detail by Hokama (in press). One of the most important innovations in this test is the use of coated sharp sticks to sample the flesh. When poked into the fish, the coating on the stick adsorbs the lipid ciguatoxin and its related polyether toxins. The stick is air dried and immersed into a fixation solution for 1 sec. The excess solution is then blotted onto tissue paper. After washing in a buffer solution, the stick is immersed in an antibody solution, blotted, and then

washed two consecutive times in buffer. Finally, the stick is immersed in a substrate solution for 10 min. Presence of ciguatoxin is indicated colorimetrically by a bluish color. The toxicity of the sample is directly related to the darkness of color. A standard color scale or colorimeter can be used to read the sample.

LEARNING HOW TO USE THE TEST

We were able to learn this test within a few hours at Hokama's laboratory at the University of Hawaii. Our chief difficulty with the test was the subjective visual method of estimating the final darkness of color in the solution. This is not a severe problem for highly toxic samples where a dramatic color change takes place. The problem is in the borderline samples, where error caused by rating the color by different observers might cause problems. We conducted the tests in an ideal laboratory environment that was air-conditioned and free from wind and sun. All of the materials were fresh. Good refrigeration was readily available and keeping the reagents at the proper temperature was not difficult. Clean glassware, a well-trained technician, and all laboratory accessories were readily at hand. This is quite different from working at a field site with reagents that have been shipped or stored for a prolonged period. The question which we address in this report is:

Can fishermen or others working under field conditions use this technique with the same degree of precision that we were able to achieve in Hokama's laboratory?

This is a very important question if the technique is to be widely employed for detecting ciguatera. It is of less value if reproducible measurements can only be made in certain laboratories. Our aim was to test fish at a remote field site, detect possible problems, and recommend changes in the technique if we ran into difficulties.

SELECTION OF FIELD SITE

We decided to run the initial trials at Midway in the NWHI. This site has experienced a series of serious ciguatera outbreaks which have led to a closing of the shallow water fishery in the region. The area is a U.S. Navy air facility. It was possible for us to take advantage of a scheduled cruise to this island by the NOAA ship Townsend Cromwell. The senior author participated in the first 3 weeks of this cruise, after which he disembarked at Midway with his equipment and test kits. He spent several days catching and testing fish prior to his return to Honolulu. There were problems with use of the kits on site. Therefore, the samples were frozen and returned to Honolulu for testing within 2 weeks. Nevertheless, a great deal was learned from the attempt to run the test in the field. Midway is the type of remote location where these kits might prove to be most useful. It is typical of many locations throughout the Pacific where the uncertainty about the occurrence of ciguatera in the locally caught fish prevents their consumption or shipment to markets.

USE OF THE TEST AT MIDWAY AND RECOMMENDED IMPROVEMENTS

Conducting the test at Midway proved to be a useful method of identifying its present shortcomings in field situations. Numerous problems presented themselves, but all were related to storage of reagents and sample processing. These problems were purely the result of doing the test in a remote location; none would have surfaced if we conducted the test in Dr. Hokama's laboratory. Each of the problems encountered can be corrected with relatively little effort. The problems encountered and recommended solutions (not in any order of importance) are as follows:

Problem 1: Unreactive fixing reagent. The test did not work initially. The problem was later traced to the fixative. The hydrogen peroxide that had been added to the fixative in the kit may have decomposed, possibly because the reagent bottle was transparent and hydrogen peroxide is unstable in light.

Recommendation: Use an opaque bottle to store fixative in kit or do not add the hydrogen peroxide until ready to start the test.

Problem 2: Inadequate materials.--It is important to develop a complete list of materials needed. Time was wasted trying to obtain suitable containers, test tube brushes, etc. The funnel and paper supplied for filtering the substrate were not adequate to handle the volume. More trials should be conducted to identify areas of difficulty.

Recommendation: Provide all needed containers and supplies in the kit. Maintain a list of all items needed so that users of kits can purchase items not supplied. A suggested list is presented in Appendix 1 of this report.

Problem 3: Poor experimental design.--The reagents in various containers were not of the proper depth to insure that the correct length of stick would be immersed.

Recommendation: Standardize the vessels included in the kit. Make certain that standard solutions will fill vessels to proper level. Standardize the length of stick covered with liquid paper. This problem seems to be a major source of variability; better quality control is needed.

Problem 4: Tipping of vessels containing reagents.--The reagent containers became unstable and spilled due to loss of support from the rapidly melting ice. Water overflow must be anticipated.

Recommendation: Well designed racks are needed to support the reagent vessels. The racks should be placed in an insulated container with a drainspout and tube that drains the melted water into a waste container. The container should have an insulated closing lid to maintain proper reagent temperature between tests.

Problem 5: Degradation of reagents.--Increasingly variable results occur as one proceeds with the test in a series of fish. The reagents seem to degrade. It became impossible to replicate tests on a single fish and on controls. Some interreagent contamination may occur with time due to ineffective blotting of the sticks between washes.

Recommendations: More effective blotting may alleviate the contamination problem. Changing washes and antibody midway through the batch of samples also appears to reduce contamination. After dilution, half of the antibody supplied in the kit can be used, and half stored in a refrigerator until needed for the second half of the samples.

Problem 6: Coagulated antibody. The antibody provided in the kit had partially coagulated in transit. Apparently this does not influence the test. One simply removes the coagulated material.

Recommendation: Include a note about this in the instruction to prevent undue concern by the person doing the test.

Problem 7: Lack of color standards.--It was very difficult to read the colors accurately due to changes in room lighting and due to the lack of a good color reference. Also, the test tubes obtained were not of uniform optical clarity.

Recommendation: Provide a small rack with comparative color standard tubes in the rack. This rack should have its own light source (uniform back lighting) so that the reference tubes and sample tubes can be accurately compared.

Problem 8: Incomplete or outdated instructions; lack of sequential instructions.--This test is undergoing very frequent modifications because it is in the developmental stage. Several modifications were made in the brief time between our laboratory training and the subsequent field evaluation, but the instructions were not modified to reflect these changes.

Recommendation: Develop complete written instructions and keep them on a word processor so that the instructions can be easily modified and updated. Be certain to modify the instructions every time that the test is modified. Develop a "trouble-shooting" appendix that covers all of the possible problems that people have experienced. Weak instructions can lead to problems with the test and this can easily be avoided. We expanded the previous instructions into a more complete set of instructions in Appendices 2 and 3 of this report.

Problem 9: Difficult to prepare reagents and conduct test due to lack of a supply of clean glassware and pipettes in a field situation.

Recommendation: Substitution of dropper bottles for pipettes has already been accomplished, allowing one to measure reagents by counting the number of drops. We have found this method to be much easier to use. We recommend the use of disposable test tubes and containers in remote field sites where it is difficult to properly wash and dry glassware.

Problem 10: Inconsistent results from use of positive and negative controls at beginning of test.--Note in Appendix 4 that the positive controls often read negative. This appeared to be a problem with the material supplied as the "positive" ciguatoxin source.

Recommendation: A faulty ciguatoxin reference sample would yield low readings. This might not have resulted in any problem in terms of test accuracy, but we lost the means of verifying the higher readings of the test. This was annoying and tended to weaken our confidence in the test. We suggest that a more reliable set of positive and negative controls be devised in the future.

Other observations: All aspects of the test are influenced by high temperature and high humidity associated with conducting the test in a non air-conditioned environment. Reagents are less stable, it takes much longer to dry the sticks after poking the fish and it is very difficult to properly dry the glassware.

SUMMARY OF LABORATORY VERSUS FIELD USE

We agree that the stick test has potential as a method for detecting ciguatoxic fish in the laboratory, but problems arise when the test is taken into the field. None of these problems appear to be insurmountable. We suggest that frequent field evaluations be conducted in conjunction with laboratory evaluations in order to eliminate problems early in development. If this test is to be useful to fishermen, it must be reliable and easy to use aboard ships or at fish markets and restaurants. Rapid results are necessary if the fish are to be sold fresh, so a field test is needed.

SUMMARY OF TEST RESULTS

All of the data taken on 239 fish at Midway are presented in Appendix 4 of this report. The toxicity results are summarized in Table 1.

Table 1.--Total numbers of fish and percent of total that tested positive, borderline, and negative for ciguatoxin at Midway.

Toxicity	Total	Percent
Positive	38	16
Borderline	101	42
Negative	98	41
No data	2	1

These results are quite consistent with previous surveys using more complicated methodology. The use of enzyme-immunoassay (EIA) in reef fish of the NWHI produced an overall positve rate of 12% for a similar group of species (Kimura et al. 1984). Another survey (Ito et al. 1984) studied deeper water species of the NWHI and yielded a positve rate of 10%, but some locations ran as high as 44%. Therefore, the data in Table 1 seem to be consistent with past results. If the stick test is comparable to past tests, then we have shown that great savings in time and money can be achieved. The relatively high rate of fish with a positive score for ciguatoxin at Midway is consistent with experience. Most reef species are not eaten at this location because of the high previous incidence of fish poisonings.

Table 2.--Summary of test results (App. 4) for those species caught in greatest number. Values above 2.5 are positive for ciguatoxin. Values of 1.5 to 2.4 are borderline (see App. 3).

Species	n	Mean	Range
Thallasoma ballieui	31	2.4	1.0-3.4
Kuhlia sandvicensis	30	1.4	0.7-2.5
Scarus perspicillatus	29	1.9	1.0-2.8
Abudefduf abdominalis	24	1.6	1.1-2.9
Kyphosus spp.	21	1.9	0.6-3.0
Acanthurus triostegus	14	1.2	0.6-2.1
Mugil cephalus	14	1.2	0.6-2.1
Chaetodon fremblii	9	1.5	0.4-2.9
Acanthurus leucopareius	8	1.2	1.0-1.8
Bodianus bilunulatus	6	1.7	1.3-2.5
Thalassoma duperreyi	6	2.2	1.2-3.1
Thalassoma umbrostigma	6	1.5	0.4-2.6
Neoniphon sammara	6	1.5	1.2-2.1

An extremely interesting observation is the high rate (42% of fish tested) in the "borderline" category. These fish could become "positive" with further ingestion of the toxin. This suggests constant production of toxin in the environment, and that very severe outbreaks of ciguatera poisonings may reflect only a slight increase in production of toxin at the lower trophic levels. Similar groups of borderline tests have been noted by others (Ito et al. 1984; Kimura et al. 1984). Populations of fish might exist in a state that is very close to being toxic, but can be eaten. A very slight environmental perturbation might tip the balance slightly in favor of toxin production, producing an extraordinary increase in rate of ciguatera poisonings. These subtleties would be very hard to document and might explain the difficulty of dealing with the ciguatera problem.

Residents of the island believe that at least three species of reef fish at Midway are free of ciguatoxin (R. Schroeder pers. commun.). Apparently this "folk wisdom" was obtained by trial and error. The mullet Mugil cephalus, the moi, Polydactylus sexfilis, and the aholehole, Kuhlia sandvicensis, are eaten regularly by some inhabitants. These three species had extremely low scores. The mullet (n = 13) had a mean score of 1.2 with a range of 0.8 to 1.6. The moi sample was rather small (n = 3), but the mean score was only 1.2 with a range of 1.0 to 1.5. The aholehole (n = 30) had a very low mean score of 1.4 with a range of 0.7 to 2.5. This agreement supports the contention that the stick test measures ciguatoxicity.

COMPARISON OF STICK TEST RESULTS TO MOUSE BIOASSAY TEST

The mouse bioassay involves direct extraction of the toxin from a sample of fish flesh of approximately 100 g and injection of the extract into a mouse. Presence of toxin in the sample will cause the death of the mouse within 24 h (Kimura et al. 1982).

Forty-six of the fish tested with the stick test were selected for analysis by the mouse bioassay. The mouse test is expensive, so we were limited on the number that could be run. We selected samples that ranged widely in toxicity as indicated by the stick test. Several species were involved. The mouse test was run blind; only a code number was provided. Data relevant to the mouse test is presented in Appendix 5 and a comparison between the mouse test and the stick test is presented in Appendix 6.

The data in Appendix 6 are very encouraging in spite of the technical problems encountered and discussed earlier. Correction of these problems will undoubtedly enhance the accuracy of the test. Nevertheless, the species that are eaten locally without fear of poisoning (M. cephalus and K. sandvicensis) tested as safe by the stick test and entirely safe by the mouse test. In some samples, the stick test gave a positive reading for a fish that subsequently did not kill a mouse. The false positive is not a problem in that it only causes one to discard a fish that might have been safe to eat. A false positive could easily be the result of okadaic acid, a nontoxic substance related to ciguatoxin. Apparent discrepancies occurred in six cases where the stick test gave a negative result, whereas the mouse test gave a positive result. The stick test is specific for

ciguatoxin but can be influenced by other toxic and nontoxic polyethers. It will not detect nonpolyether toxins. We believe there are two possible explanations for these discrepancies. As mentioned earlier, we did have some problems with reagent stability and might have failed to get a reaction due to this factor. A second interpretation is that the stick test fails to read maitotoxin, scaritoxin, or a related toxin that killed the mice. This problem is in need of further study.

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Appendix 1.--Suggested complete list of materials.

Deep tray with rack to hold reagents
Coated bamboo sticks (see App. 2)
Reagents (see App. 2)
Data sheets
Knife
Disposible pipettes
Filter paper
Filter funnel
Two 200 ml beakers, graduated
One vessel to hold substratum solution in ice bath
Disposible test tubes
Test tube racks
Toothpicks to scrape 4-chloro-1-napthol from wax paper
Parafilm
Indelible pens
Control specimen
Timer (seconds)
Kimwipes
Paper towels
Labels
Background board for consistent color scaling
Detergent
Bottle brush

Items needed on site: refrigerator, freezer, ice supply, freshwater, sink for washing, oven for drying glassware (kitchen oven will suffice), and table.

Appendix 2.--Suggested recipes for test solutions.

The following formulas yield enough test solution for 30 fish (5 sticks each). Once made up, the solutions must be kept under refrigeration or packed in ice. They are good for only 3 h and must be discarded after that time.

(We recommend use of simplified one-word terms in referring to test solutions for clarity in the instructions.)

The following reagents are needed to prepare the test solutions: ethanol, methanol, distilled water, 30% hydrogen peroxide, Tris Buffer-A, Tris Buffer-B, sheep-anti-ciguatoxin-horseradish peroxidase (sheep-anti-CTX-HRP), 4-chloro-1-naphthol.

Reagent storage: Hydrogen peroxide decomposes in light and should be stored under refrigeration in an opaque bottle. Concentrated sheep-anti-CTX-HRP must be kept at -20° to -100°C. The 4-chloro-1-naphthol and the control material must be kept frozen. Tris Buffer-A and -B must be kept under refrigeration.

Preparation of the four test solutions:

1. Fixative: Prepare by mixing 18 drops of 30% hydrogen peroxide in 24.75 ml of absolute methyl alcohol. The resulting 0.3% solution of hydrogen peroxide in methyl alcohol is prepared fresh daily and must be kept in an ice bath during the test procedure.

2. Antibody: Prepare by mixing premeasured sheep-anti-CTX-HRP to 10 ml Tris Buffer-A (1:200 dilution). The antibody must be kept in an ice bath.

3. Buffer: Use Tris Buffer-B. This solution must be stored in an ice bath.

4. Substrate: First, dilute 2 drops of 30% hydrogen peroxide with 18 drops of distilled water. This will yield a 3% solution of hydrogen peroxide. Add 12 drops of 3% hydrogen peroxide to 100 ml Tris Buffer-B in 200 ml flask, set aside. Finally, add 12 drops ethanol to 4-chloro-1-naphthol in second 200 ml flask. Then add hydrogen peroxide/Tris Buffer-B solution to 4-chloro-1-naphthol/ethanol solution. Shake vigorously, then filter into final substrate solution vessel. Keep this solution in an ice bath.

Appendix 3.--Suggested serial instructions for test procedure.

General Procedures

1 Preparation (30-45 min).

1.1 Prepare sample sticks following steps 1.6 to 1.6.6.

1.2 Prepare control sticks following steps 1.7 to 1.7.3

1.3 While control sticks are drying, spread each set of sample sticks into five labeled test tubes so there is only one stick per test tube.

1.4 Mix reagents (45 min) while sticks are drying (Appendix 2). Use only one half of antibody solution, retain second half to replace antibody midway through the samples.

1.5 Set reagents in ice bath.

1.6 Sample stick preparation (90 min/30 fish).

1.6.1 Make several slits in the skin of the fish in areas of firm muscle.

1.6.2 Insert skewered end of stick into fish flesh and rotate for 1 sec.

1.6.3 Do not poke stick into visceral cavity, this may give a high reading.

1.6.4 Remove stick, place each stick in one labeled test tube.

1.6.5 Repeat for all fish to be tested.

1.6.6 Place all sets (set = 5 sticks/fish) where they will dry. In humid environments, it is best to use a frost-free refrigerator.

1.7 Control stick preparation (5 min/5 pair).

1.7.1 Thaw and poke each control (positive and negative) with 5 sticks.

1.7.2 Label positive and negative sticks and place each in an appropriately marked test tube.

1.7.3 Set sticks aside to dry.

2 Procedure for running control and sample sticks.

2.1 Immerse sticks, skewered end, into fixative for 1 sec, blot excess liquid onto absorbent paper towel.

2.2 Wash sticks thoroughly in buffer bath 1 by shaking it gently for 5 sec, blot excess buffer onto absorbent paper towel.

2.3 Immerse sticks in antibody solution for 30 sec, blot excess onto absorbent paper towel. While the sticks are soaking in the antibody solution, put 7 drops of substrate solution into each of their test tubes.

2.4 Wash in buffer bath 2, shaking sticks gently in buffer for 15 sec, blot excess.

2.5 Wash in buffer bath 3, shaking sticks gently in buffer for 15 sec, blot excess.

2.6 Immerse each stick in its test tube containing 7 drops of substrate at room temperature. Shake sticks and solution from side to side with gentle to moderate motion for 5 sec to thoroughly soak sticks with substrate.

2.7 Allow sticks to stand for 10 min, read color according to chart.

2.8 Reference color chart:

Color	Numerical Score	Toxicity
None	0	Negative
Slight bluish	1.0-1.4	Negative
Lightly bluish-purple	1.5-2.0	Borderline
Moderately bluish-purple	2.0-2.4	Borderline
Moderate to dark bluish-purple to purple	2.5-5.0	Positive

3 Run and read controls following steps 2 to 2.8 (15 min).

3.1 Proceed unless more than two show false negative. In this case, check washes for clarity and mix a few drops of antibody solution with a few drops of substrate solution. If this does not turn dark purple, replace substrate solution. Mix again, if still no dark purple color, replace antibody.

4 Testing samples (180 min).

4.1 Now that controls work you can begin running the dry sample sets.

4.2 Run set (or portion) following steps 1.7 to 2.7.

4.3 Read set (or portion) according to step 2.8.

4.4 After 15 sets or 1.5 h, you must replace rinses and antibody with second half set aside at start of test.

4.5 Repeat 4.2 and 4.3.

5 Clean up (45 min).

Appendix 4.--Data on Midway Island fishes.

I.D.[1]	Test subject	Length (cm)	Weight (kg)	Sex	Mean score	Range
	Positive control (initial)				3.0	3.0-3.0
	Negative control (initial)				2.5	2.5-2.5
1	Scarus perspicilla	42.3	2.77	M	2.7	2.5-3.0
2	do	46.8	3.83	M	2.2	1.0-3.0
3	do	45.6	3.30	M	2.4	2.0-3.0
4	do	32.7	1.26	F	1.7	1.0-2.0
5	do	31.2	1.20	F	2.4	1.0-4.0
6	do	30.4	1.06	F	2.4	1.5-3.0
7	do	36.3	1.59	F	2.2	2.0-3.0
8	do	30.8	1.11	F	2.5	2.5-2.5
9	do	31.1	1.12	F	1.8	1.0-2.5
10	do	25.7	0.61	F	2.0	1.0-3.0
11	Bodianus bilunulatus	31.9	1.10	M	2.2	2.0-2.5
12	do	34.0	1.34	M	2.5	2.0-4.0
13	Kyphosus spp.	35.9	1.53	--	2.6	2.5-3.0
14	do	21.0	0.26	--	2.3	2.0-3.0
15	Abudefduf abdominalis	16.7	0.17	--	1.9	1.0-2.5
16	do	15.7	0.16	--	1.8	1.0-2.5
17	Zebrasoma veliferum	25.2	0.57	--	1.7	1.0-2.0
18	do	25.1	0.58	--	1.2	1.0-2.0
19	Parupeneus multifasciatus	20.6	0.25	--	2.2	2.0-2.5
20	Thalassoma ballieui	27.8	0.63	--	2.2	1.0-3.0
21	do	25.5	0.47	--	2.0	2.0-2.0
22	do	20.8	--	--	2.0	2.0-2.0
23	do	21.2	0.23	--	2.2	2.0-2.5
24	Seriola dumerili				2.0	2.0-2.0
	Positive control (initial)				2.8	1.0-3.5
	Negative control (initial)				1.8	1.0-2.0
25	Kyphosus spp.	20.7	0.24	--	2.5	2.0-3.0
26	do	23.9	0.39	M	2.0	1.5-2.0
27	do	26.3	0.61	M	2.1	1.5-2.5
28	do	26.5	0.63	M	2.0	1.5-2.5
29	do	28.6	0.79	F	1.4	1.0-2.0
30	do	21.3	0.26	--	1.6	1.0-3.0
	Positive control (monitor)				1.6	1.0-3.0
	Negative control (monitor)				0.0	0.0-0.0
31	Kyphosus spp.	22.1	0.35	--	2.0	1.0-3.0
32	do	23.0	0.37	--	3.0	1.5-4.0
33	Thalassoma ballieui	29.5	0.63	M	1.8	1.0-3.0
34	do	26.6	0.54	M	1.1	1.0-1.5
35	do	26.0	0.47	--	2.0	2.0-2.0

Appendix 4.--Continued.

I.D.[1]	Test subject	Length (cm)	Weight (kg)	Sex	Mean score	Range
36	Thalassoma ballieui	20.5	0.18	F	2.0	2.0-2.0
37	do	20.0	0.18	F	2.0	2.0-2.0
38	do	20.0	0.20	M	2.0	2.0-2.0
	Positive control (terminal)				2.0	2.0-2.0
	Negative control (terminal)				2.0	2.0-2.0
	Positive control (initial)				1.3	0.0-2.5
	Negative control (initial)				1.3	0.5-2.0
	Positive control (initial)				0.0	0.0-0.0
	Negative control (initial)				1.2	1.0-1.5
	Positive control (initial)				3.2	2.5-4.0
	Negative control (initial)				2.7	2.0-3.0
39	Thalassoma ballieui	17.7	0.13	M	2.9	2.5-3.0
40	do	22.1	0.26	-	2.7	2.0-3.0
41	do	27.5	0.52	M	3.0	3.0-3.0
42	do	26.0	0.47	M	3.4	3.0-4.0
43	do	24.3	0.34	M	2.5	2.0-3.0
44	do	26.1	0.50	M	3.0	3.0-3.0
45	do	23.5	0.34	M	3.3	3.0-3.5
46	do	21.8	0.24	-	2.9	2.5-3.0
47	do	23.0	0.28	F	1.7	1.0-2.0
48	do	21.5	0.23	F	2.2	1.0-3.0
49	do	20.1	0.21	F	2.5	2.0-3.0
50	do	18.7	0.15	-	2.1	1.0-3.0
51	do	18.8	--	-	2.8	2.0-3.0
52	do	18.9	0.16	-	3.2	2.5-5.0
53	do	17.9	0.13	-	2.6	2.0-3.0
54	do	17.5	0.12	-	3.0	3.0-3.0
55	do	16.4	0.10	-	2.4	2.0-3.0
56	do	14.7	0.07	-	2.6	2.0-3.0
57	do	15.4	0.07	-	3.2	3.0-4.0
58	Acanthurus triostegus	12.8	0.08	F	1.2	1.0-2.0
59	Abudefduf abdominalis	15.9	0.15	M	2.9	2.0-3.5
60	do	16.7	0.19	F	2.4	1.0-3.0
61	Gymnothorax steindachneri	37.0[2]	0.09	-	2.5	2.0-3.0
62	Myripristis argyromus	21.3	0.25	F	2.8	2.0-3.5
63	Thalassoma umbrostigma	25.4	--	F	1.8	1.0-2.0
64	do	16.0	0.11	F	1.9	1.0-2.5
65	Thalassoma duperreyi	14.7	0.10	-	1.8	1.0-2.0
66	do	17.6	0.11	M	3.0	2.5-4.0
67	do	14.4	0.08	M	3.1	2.5-4.0

Appendix 4.--Continued.

I.D.[1]	Test subject	Length (cm)	Weight (kg)	Sex	Mean score	Range
68	Bodianus bilunulatus	33.0	1.22	F	2.3	2.0-2.5
69	do	39.7	2.22	M	No data	
	Positive control (initial)				2.5	2.0-3.0
	Negative control (initial)				1.8	1.0-2.5
70	Scarus perspicillatus	38.8	2.50	M	2.8	2.5-3.0
71	do	37.8	46.4	M	2.3	1.0-3.0
72	do	35.9	1.55	F	1.2	1.0-1.5
73	do	34.5	1.66	F	1.4	1.0-2.0
74	do	37.7	1.75	F	1.7	1.0-2.5
75	do	29.4	1.02	F	1.6	1.0-2.0
76	do	35.7	1.77	M	2.5	2.0-3.0
77	do	36.7	1.60	M	1.8	1.0-2.0
78	Kyphosus spp.	18.3	0.18	M	1.8	1.0-2.5
79	Cirrhites pinnulatus	13.0	0.10	F	2.0	2.0-2.0
80	Acanthurus triostegus	11.8	0.08	F	1.4	1.0-2.0
81	Chaetodon trifasciatus	12.3	0.08	M	2.3	1.0-3.0
82	Mulloidichthys auriflamma	23.6	0.25	F	1.6	1.0-2.0
83	Thalassoma duperreyi	14.0	0.09	F	1.4	1.0-2.0
84	do	15.7	0.08	M	2.7	2.0-3.0
85	do	15.4	0.09	M	1.2	1.0-2.0
86	Abudefduf abdominalis	12.6	0.09	M	2.1	2.0-2.5
87	do	15.5	0.19	M	1.4	1.0-2.0
88	do	16.0	0.20	M	1.6	1.0-2.0
89	Acanthurus leucopareius	17.2	0.34	M	1.4	1.0-2.0
90	do	17.2	0.25	F	1.6	1.0-2.5
91	Thalassoma ballieui	26.0	0.43	M	1.6	1.0-2.0
92	do	26.5	0.45	M	1.0	1.0-1.0
93	Scarus perspicillatus	36.0	1.48	M	2.0	2.0-2.0
94	do	36.6	1.75	M	1.5	1.0-2.0
95	do	37.3	1.75	M	2.7	2.0-3.0
96	do	38.8	1.23	M	1.6	1.0-2.0
97	do	39.4	2.26	M	1.4	1.0-2.0
98	do	42.0	2.44	M	1.0	1.0-1.0
99	Carcharhinus galapagensis	84.0		F	1.7	1.5-2.0
100	Kuhlia sandvicensis	17.1	0.13	M	1.5	1.0-2.0
101	do	18.8	0.19	F	1.8	1.0-2.5
	Positive control (initial)				2.5	2.0-3.0
	Negative control (initial)				1.8	1.0-2.5
102	Kuhlia sandvicensis	18.4	0.16	M	1.6	1.0-2.0
103	do	17.0	0.13	F	1.4	1.0-2.0
104	do	18.1	0.16	F	1.2	1.0-1.5

Appendix 4.--Continued.

I.D.[1]	Test subject	Length (cm)	Weight (kg)	Sex	Mean score	Range
105	Kuhlia sandvicensis	18.2	0.12	M	1.5	1.0-2.0
106	do	17.4	0.16	F	1.4	1.0-2.0
107	do	20.1	0.23	F	1.2	1.0-2.0
108	do	19.9	0.24	F	2.0	2.0-2.0
109	do	17.8	0.17	F	1.8	1.0-2.0
110	do	16.8	0.14	F	1.2	1.0-2.0
111	do	19.0	0.19	F	1.4	1.0-2.0
112	do	18.0	0.16	F	1.0	1.0-1.0
113	do	16.6	0.12	M	1.4	1.0-2.0
114	do	15.0	0.10	F	1.5	1.5-1.5
115	do	17.0	0.15	F	1.6	1.0-2.0
116	Mulloidichthys auriflamma	15.1	0.09	M	1.4	1.0-1.5
117	Polydactylus sexfilis	15.8	0.10	-	1.5	1.5-1.5
118	do	16.2	0.10	-	1.0	1.0-1.0
119	Kyphosus spp.	13.6	0.10	M	1.1	1.0-1.5
120	Mugil cephalus	15.4	0.09	F	1.0	1.0-1.0
121	do	19.2	0.18	F	1.2	1.0-2.0
122	do	19.8	0.17	-	1.3	1.0-1.5
123	do	21.5	0.23	-	1.2	1.0-2.0
124	do	19.1	0.15	-	1.6	1.0-2.0
125	do	22.4	0.24	-	1.0	1.0-1.0
126	do	22.1	0.24	M	1.0	1.0-1.0
127	do	23.1	0.29	F	1.1	1.0-1.5
128	do	19.3	0.17	F	1.4	1.0-2.0
129	do	20.6	0.22	-	1.0	1.0-1.0
130	do	19.5	0.19	M	1.5	1.0-2.0
131	do	22.5	0.26	-	1.4	1.0-2.0
132	do	20.4	0.19	-	0.8	0.0-1.0
133	Polydactylus sexfilis	15.7	0.10	M	1.0	1.0-1.0
134	Abudefduf abdominalis	9.5	0.05	F	1.5	1.0-2.0
135	Acanthurus triostegus	10.0	0.07	-	1.1	1.0-1.5
136	do	10.6	0.08	F	1.1	1.0-1.5
137	do	15.0	0.19	-	2.1	1.5-2.5
138	Kyphosus spp.	12.4	0.06	-	2.2	2.0-2.5
	Positive control (initial)				2.0	2.0-2.0
	Negative control (initial)				2.0	2.0-2.0
139	Kyphosus spp.	12.6	0.08	M	2.6	2.5-3.0
140	do	15.9	0.12	-	1.3	1.0-2.0
141	do	16.9	0.16	F	1.8	1.5-2.0
142	do	14.2	0.08	M	3.0	3.0-3.0
143	do	17.5	0.18	-	1.0	1.0-1.0
144	do	19.7	0.29	F	2.0	2.0-2.0

Appendix 4.--Continued.

I.D.[1]	Test subject	Length (cm)	Weight (kg)	Sex	Mean score	Range
145	Kuhlia sandvicensis	17.5	0.16	M	1.0	1.0-1.0
146	do	16.8	0.14	M	1.4	0.0-2.0
147	do	18.4	0.20	F	1.2	1.0-2.0
148	do	16.7	0.14	M	1.0	1.0-1.0
149	do	18.6	0.19	M	2.2	2.0-2.5
150	do	16.2	0.13	F	0.9	0.0-1.5
151	do	18.1	0.19	M	0.7	0.5-1.0
152	Parupeneus multifasciatus	19.6	0.19	F	0.9	0.5-2.0
153	do	11.5	0.04	M	0.9	0.0-2.0
154	Chaetodon fremblii	12.2	0.07	F	2.1	1.0-3.0
155	do	12.1	0.07	M	1.6	1.0-2.0
156	do	10.9	0.05	F	1.0	1.0-1.0
157	do	11.4	0.05	F	0.8	0.0-1.0
158	do	11.2	0.04	F	1.0	1.0-1.0
159	do	10.0	0.04	M	1.3	1.0-2.0
160	do	10.2	0.04	F	0.4	0.0-1.0
161	Kyphosus spp.	27.4	0.70	M	1.4	1.0-2.0
162	do	24.4	0.47	M	0.6	0.5-1.0
163	Zanclus cornutus	15.9	0.24	F	0.6	0.5-1.0
164	Acanthurus triostegus	14.3	0.09	M	1.0	1.0-1.0
164	do	13.3	0.11	M	0.9	0.0-1.5
165	do	12.9	0.10	F	1.0	1.0-1.0
166	do	13.5	0.12	F	1.6	1.0-2.5
167	do	12.3	0.09	F	1.1	1.0-1.5
168	Neoniphon sammara	16.7	0.12	F	1.2	1.0-1.5
169	do	16.5	0.12	F	1.4	1.0-2.0
170	do	17.8	0.14	M	1.3	1.0-2.0
171	do	19.4	0.16	F	2.1	1.5-2.5
172	do	19.0	0.15	F	1.4	1.0-2.0
173	do	17.5	0.12	-	1.5	1.0-2.5
	Positive control (initial)				1.3	1.0-2.0
	Negative control (initial)				1.5	1.0-2.5
174	Neoniphon sammara	17.5	0.12	--	1.5	1.0-2.5
175	Cirrhites pinnulatus	14.5	0.10	F	1.0	1.0-1.0
176	do	15.9	0.19	F	1.6	1.0-2.0
177	Thalassoma umbrostigma	15.7	0.07	F	2.6	2.0-3.0
178	do	17.3	0.12	F	0.4	0.0-0.5
179	Acanthurus leucopareius	16.3	0.24	F	1.4	1.0-2.0
180	do	16.3	0.20	M	1.2	1.0-1.5
181	do	13.0	0.10	F	No data	
182	do	14.2	0.14	F	1.0	0.0-1.5
183	do	14.2	0.13	F	1.4	1.0-2.0
184	do	15.9	0.22	F	1.8	1.0-2.0

Appendix 4.--Continued.

I.D.[1]	Test subject	Length (cm)	Weight (kg)	Sex	Mean score	Range
185	Fistularia petimba	77.5	0.32	F	1.6	1.0-2.0
186	Chaetodon unimaculatus	15.1	0.20	F	1.2	1.0-2.0
187	Chaetodon ornatissimus	18.2	0.35	F	1.0	1.0-1.0
188	Bodianus bilunulatus	37.2	1.79	F	1.8	1.0-2.0
189	do	39.6	2.14	M	1.3	1.0-1.5
190	Scarus perspicillatus	40.4	2.37	F	1.2	1.0-2.0
191	do	40.2	2.31	M	1.4	1.0-2.0
192	do	36.9	1.74	M	1.7	1.0-2.5
193	do	33.5	1.46	M	1.0	1.0-1.0
194	do	40.4	2.25	F	1.5	1.0-2.0
195	Chaetodon fremblii	10.6	0.05	--	2.9	2.5-3.0
196	do	12.4	0.08	--	2.6	2.5-3.0
197	do	9.0	0.03	--	2.5	2.0-3.0
198	do	14.2	0.11	--	0.9	0.0-1.5
199	Flammeo sammara	14.0	0.06	--	1.4	1.0-2.0
200	Kuhlia sandvicensis	15.6	0.10	--	2.0	1.0-3.0
201	do	13.2	0.06	--	1.4	1.0-2.0
202	do	14.1	0.07	--	1.2	0.0-2.0
203	do	16.1	0.10	--	1.0	0.0-2.0
204	do	14.4	0.08	M	1.7	0.0-3.0
205	do	13.4	0.06	--	1.0	1.0-1.0
206	do	16.2	0.10	M	2.5	1.5-3.0
	Positive control (initial)				0.8	0.0-3.0
	Negative control (initial)				0.7	0.0-3.0
207	Acanthurus triostegus	13.0	0.10	--	0.6	0.0-2.0
208	do	16.3	0.15	--	0.6	0.0-1.0
209	do	14.3	0.14	--	1.3	0.0-2.0
210	do	13.5	0.11	--	1.3	0.0-2.0
211	Abudefduf sordidus	14.0	0.15	--	2.0	1.0-3.0
	Positive control (monitor)				1.1	0.0-2.5
	Negative control (monitor)				2.3	1.0-3.0
212	Abudefduf sordidus	13.2	0.13	--	2.0	2.0-2.0
213	do	11.9	0.10	--	1.6	1.0-2.0
214	do	11.1	0.08	--	1.4	1.0-2.0
215	Abudefduf abdominalis	10.4	0.05	--	2.1	1.0-3.0
216	do	10.9	0.06	--	2.1	1.0-2.5
217	do	11.3	0.07	--	1.4	0.0-3.0
218	do	11.2	0.07	--	1.5	1.0-2.0
219	do	11.0	0.06	--	2.3	0.0-3.0
220	do	11.1	0.06	--	1.5	0.0-2.5
221	do	10.3	0.05	--	1.4	1.0-3.0

Appendix 4.--Continued.

I.D.[1]	Test subject	Length (cm)	Weight (kg)	Sex	Mean score	Range
222	Abudefduf abdominalis	11.2	0.07	--	1.1	0.0-2.5
223	do	10.7	0.05	--	1.6	1.0-2.0
224	do	12.1	0.07	--	1.1	0.0-2.0
225	do	10.1	0.04	--	1.4	1.0-2.5
226	do	10.5	0.05	--	1.4	0.0-2.0
227	do	10.6	0.05	--	1.4	0.0-2.0
228	do	10.2	0.05	--	1.2	1.0-1.5
229	do	11.3	0.06	--	1.2	1.0-1.5
230	do	10.7	0.06	--	1.1	1.0-1.5
231	Gymnothorax steindachneri	51.9	0.33	--	1.8	1.0-2.5
232	do	48.5	0.17	--	1.5	1.0-2.0
233	Mulloides vanicolensis	22.1	0.19	F	1.1	0.0-2.0
234	Thalassoma umbrostigma	16.7	0.10	--	1.2	1.0-1.5
235	do	15.3	0.08	--	0.9	0.0-1.5
236	Gymnothorax steindachneri	49.9	0.25	--	2.7	2.5-3.0
237	Conger cinereus	111.2	2.87	M	2.3	1.5-3.0
238	do	98.2	1.65	F	2.0	1.0-2.5
239	Carangoides ferdau	59.8	3.39	F	1.0	1.0-1.0

[1]I.D. = identification.
[2]Tail length.

Appendix 5.--Data for mouse bioassay: yields of lipid extract, dose per mouse given intraperitoneally and response.

Mouse scores:

1 to 2 = Recovery of mouse within 2 to 3 h after injection.
3 = Recovery of mouse within 4 to 6 h after injection.
4 = Mouse dead after 20 to 24 h.
5 = Mouse dead within 3 h after injection. In this study most of the "5" scores were dead within 90 min of injection.

Blind code No.	Sample weight (g)	Extract yield (mg extract per g of fish)	Mouse dose (mg extract per kg of mouse)	Mouse response
1	90	0.64	2,306	2
2	80	0.49	1,774	2
3	62	0.50	1,229	2
4	168	0.59	4,108	2
5	181	0.43	3,513	3
6	95	0.41	1,875	2
7	118	0.62	3,229	3
8	50	0.44	937	2
9	92	0.34	1,233	2
10	60	0.40	1,068	2
11	85	0.68	2,706	2
12	118	0.45	2,346	2
13	125	1.76	2,203, 1,969, 4,717	5, 5, 5
14	127	1.78	2,100, 4,405	5, 5
15	113	1.95	2,084, 4,348	5, 5
16	113	1.91	2,033, 4,066	4, 5
17	113	1.89	1,866, 4,149	4, 4
18	150	1.50	1,976, 4,032	3, 2
19	105	0.40	1,774	5
20	87	2.20	2,326, 4,545	2, 1
21	122	1.59	2,000, 4,167	1, 1
22	60	0.44	1,123	5
23	80	2.27	2,083, 3,333	5, 5
24	118	1.68	2,000, 3,802	5, 4
25	88	0.63	2,238	2
26	111	0.48	1,989	4
27	64	0.53	1,507	5
28	72	0.68	2,004	4
29	52	0.71	1,574	4
30	40	0.89	1,350	1
31	67	0.44	1,287	4
32	112	0.94	4,468	2
33	73	0.84	2,452	1
34	73	0.42	1,271	2
35	60	0.42	984	2

Appendix 5.--Continued.

Blind code No.	Sample weight (g)	Extract yield (mg extract per g of fish)	Mouse dose (mg extract per kg of mouse)	Mouse response
36	72	0.47	1,421	1
37	44	0.48	848	1
38	60	0.63	1,563	1
39	88	0.50	1,561	1
40	62	0.48	1,200	1
41	78	0.45	1,509	2
42	63	0.53	1,476	1
43	70	1.16	3,636	1
44	53	1.79	3,602	1
45	60	0.53	1,555	5
46	136	0.49	2,523	1

Appendix 6.--Results of blind comparison of stick test and mouse bioassay. Stick test scores of 2.5 and above indicate that the fish is unsafe for human consumption. We consider mouse test scores of 4-5 to indicate severely toxic fish. Scores that indicate the fish is toxic are designated with an asterisk (*). The following table compares the stick score with the mouse score.

Blind code No.	I.D.[1] No.	Species	Stick score	Mouse score	Agree	False negative	False positive
1	13	Kyphosus spp.	2.6*	2.0			x
2	14	Do	2.3	2.0	x		
3	25	do	2.5*	2.0			x
4	27	do	2.1	2.0	x		
5	29	do	1.4	3.0	x		
6	30	do	1.6	2.0	x		
7	32	do	3.0*	3.0			x
8	143	do	1.0	2.0	x		
9	161	do	1.4	2.0	x		
10	162	do	0.6	2.0	x		
11	1	Scarus perspicillatus	2.7*	2.0			x
12	8	do	2.5*	2.0			x
13	70	do	2.8*	5.0*	x		
14	72	do	1.2	5.0*		x	
15	76	do	2.5*	5.0*	x		
16	95	do	2.7*	4.5*	x		
17	98	do	1.0	4.0*		x	
18	190	do	1.2	2.5	x		
19	191	do	1.4	5.0*		x	
20	193	do	1.0	1.5	x		
21	33	Thalassoma ballieui	1.8	1.0	x		
22	34	do	1.1	5.0*		x	
23	41	do	3.0*	5.0*	x		
24	42	do	3.4*	4.5*	x		
25	43	do	2.5*	2.0			x
26	44	do	3.0*	4.0*	x		
27	45	do	3.3*	5.0*	x		
28	47	do	1.7	4.0*		x	
29	49	do	2.5*	4.0*	x		
30	52	do	3.2*	1.0			x
31	91	do	1.6	4.0*		x	
32	92	do	1.0	2.0	x		

Appendix 6.--Continued.

Blind code No.	I.D.[1] No.	Species	Stick score	Mouse score	Agree	False negative	False positive
33	107	Kuhlia sandvicensis	1.2	1.0	x		
34	108	do	2.0	2.0	x		
35	111	do	1.4	2.0	x		
36	147	do	1.2	1.0	x		
37	149	do	2.2	1.0	x		
38	151	do	0.7	1.0	x		
39	125	Mugil cephalus	1.0	1.0	x		
40	126	do	1.0	1.0	x		
41	127	do	1.1	2.0	x		
42	129	Do.	1.0	1.0	x		
43	12	Bodianus bilunulatus	2.5*	1.0			x
44	59	Abudefduf abdominalis	2.9*	1.0			x
45	62	Myripristis argyromus	2.8*	5.0*	x		
46	236	Gymnothorax steindachneri	2.7*	1.0			x

[1]I.D. = identification.